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ISOLATION AND PURIFICATION OF ANTIBODIES TO RATTLESNAKE VENOM BY AFFINITY CHROMATOGRAPHY

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The present therapeutic modality for treatment of Crotalidae envenomation in the United States involves the intravenous administration of equine source Antivenin (Crotalidae) Polyvalent (ACP). Two basic problems are associated with the use of ACP. Firstly, it is difficult to determine the amount of ACP required to mitigate the venom effects in a patient and thus large amounts of horse protein may need to be administered. Secondly, both immediate and delayed sensitivity reactions to horse serum and the treatment of patients known to be sensitive to horse proteins present a problem.

Some attempt has been made to circumvent the use of horse serum in sensitive patients by preparing antivenins in other animals (1). Also, some critical care units have allowed for the treatment of horse serum sensitive individuals with ACP under intensive monitoring and support (2). However, the need for a more highly purified immunopharmacologic agent devoid of sensitivity reactions has become evident. The present report deals with investigations into the isolation and purification of antibodies from ACP by affinity chromatographic techniques and pharmacologic studies on these purified antibodies. Finally, attention was given to the problem of anaphylactic reactions to the purified antivenin.

METHODS: Affinity chromatography using acrylamide gel was employed to isolate and purify antibodies to four rattlesnake venoms: *Crotalus atrox*, *C. adamanteus*, *C. scutulatus scutulatus* and *C. viridis helleri*. An individual column was prepared with a separate venom as affinant. Each column was prepared by dissolving 100 mg of lyophilized venom in 10 ml of acrylamide monomer (16% acrylamide, 4% N,N-methylenebisacrylamide) in phosphate buffered saline, pH 7.4, in a small beaker. Polymerization of the venom-acrylamide mixture was achieved by addition of 500 µl of 0.4% ammonium persulfate in water and 50 µl of TEMED (N,N,N-N-tetramethylethylenediamine). The mixture was well mixed and water was layered over the surface to exclude oxygen. The venom-acrylamide mixture gelled in 10 min and then was triturated by forcing broken pieces through a plastic syringe fitted with successively smaller needles from 18 gauge to 21 gauge. Phosphate buffered saline (PBS) was added to the syringes to facilitate gel pulverization. This process was repeated until the gel easily passed through the 21 gauge needle. At this point, the venom-acrylamide gel was reduced to a gritty consistency. The pulverized gel was defined 5-6 times with PBS and packed by gravity into columns 1 cm x 20 cm. The venom-acrylamide columns were washed with alternating cycles of PBS and 0.1 M glycine, pH 2.5 (0.1 M glycine, 0.154 M NaCl, pH adjusted with HCl) until a steady baseline was obtained by monitoring the

effluent at 280 nM with a spectrophotometer. The column was returned to pH 7.4 with PBS and was then ready for use.

Commercial Antivenin (Crotalidae) Polyvalent diluted 10 ml was applied to each column. A column flow rate of 0.5 ml/min was controlled with a pump and protein detection of the effluent was monitored with a spectrophotometer at 280 nM. The initial peak eluted with PBS consisted of extraneous protein. After the effluent returned to baseline, the solvent was changed to 0.1 M glycine, pH 2.5 and a second peak was eluted which consisted of purified antibody (SRA). This effluent was collected separately. The column was then restored to pH 7.4 with PBS for reuse. The pH of the antibody effluent was adjusted to 7.4 with TRIS buffer. The isolated, purified antibody effluent was dialyzed against distilled water overnight at 10°C, lyophilized and stored at -20°C. Purified antivenin was isolated to each of the four venoms by acrylamide affinity chromatography as described.

Microbiuret protein assays were performed for each antivenin following dialysis and lyophilization and also for the lyophilized product (Table 1).

The specificity of the purified, isolated antibodies for *Crotalus* venom compared to the original antivenin was determined by immunoelectrophoresis of each specific venom developed with both the specific isolated SRA and the material (Fig. 1). The purity of the isolated SRA was demonstrated by immunoelectrophoresis of both isolated antibodies and the antivenin developed with anti-horse serum (Fig. 2).

The intravenous LD_{50} for each venom studied was determined and multiples of this were mixed with two to ten times the same amounts of purified SRA, ACP or bovine serum albumin (BSA). Individual solutions of venom purified antibody, venom ACP and venom BSA were allowed to stand for 30 min before use in lethality determinations. Swiss-Webster mice, weighing 20-26 g, were administered the individual solutions by tail vein in volumes less than 100 µl. Results were interpreted at the end of 24 h. Each purified antibody and the ACP was tested against each separate venom, using BSA as a control. Tables 2 and 3 list the results of two of those studies.

The ability of the purified antibody to prevent fibrinogen clotting induced by *C. adamanteus* venom was studied by the method of Bajwa & Markland (3). The purified antibody was compared to ACP and to a control (Table 4).

Human embryonic lung cell cultures were incubated for 30 min with saline or human serum and with varying doses of *C. v.h.* venom. The tissue damage was recorded on a scale of 0 (no damage) to 4+ (complete cellular destruction and disruption). A second group of cultures was then incubated with varying amounts of the venoms of *C.* ad., *C. vh.* and *C.* s.s. according to the method of Wingert (4). The microgram amount of the purified antibodies of *C. v.h.*, *C.* ad. and *C.* s.s. to prevent cytotoxicity was determined for 20 µg of *C. v.h.* venom, 50 µg of *C.* ad. venom and 60 µg of *C.* s.s. venom, respectively. In addition, the microgram amount of ACP needed to prevent cytotoxicity from *C. v.h.* venom (20 µg) was determined (Table 5).

The ability of the purified SRA to prevent or reduce subcutaneous hemorrhage was compared to the ACP, with BSA as a control. Various amounts of venom from one tenth the intravenous LD_{50} were mixed with purified SRA, ACP and BSA in amounts two to ten times that of the venom. These solutions were allowed to stand 30 min and then injected subcutaneously over the lateral dorsal area in mice. Two hundred mice were employed in this study. Results were interpreted at 24 h

by noting the size of the hemorrhagic areas (Fig. 3).

The hypersensitivity studies were conducted in two separate experiments. The first preliminary investigation involved sensitizing guinea pigs to ACP and challenging with purified SRA, ACP or BSA. The second investigation involved assaying the release of histamine from leukocytes of an individual sensitive to horse serum by the method of Lichtenstein & Osler (5).

RESULTS: The specificity of the purified SRA was demonstrated by immunoelectrophoresis of individual venoms with the separate purified antivenins and the ACP. This revealed multiple precipitin bands with the SRA and very few precipitin bands with the ACP (Fig. 1). This was to be expected because of the concentration of specific antibodies to the venom antigens. Fig. 2 demonstrates the relative purity of the isolated SRA as compared to ACP, when both are immunoelectrophoresed and developed with rabbit anti-horse serum. The purified SRA shows a single dense precipitin band consistent with the immunoglobulin portion of horse serum, whereas the ACP demonstrates multiple bands consistent with albumin and other horse proteins. It is evident that the extraneous protein, including albumin, has been eliminated from the SRA product.

Table 2 shows the results of injecting two times the LD_{50} of *C. ad.* venom mixed with four times the weight of one LD_{50} of each species specific purified SRA, ACP and BSA. Table 3 shows the results of injection of two times the LD_{50} of *C. v.h.* venom with five times the weight of one LD_{50} of each species specific purified SRA, ACP and BSA. In each case, the species specific antivenin provided almost complete protection against lethality to its specific venom. There was some degree of cross protection provided by the *C. ad.* purified SRA when studied against the *C. v.h.* venom (Table 3).

Fibrinogen clotting results (Table 4) demonstrated protection against clotting by both *C. v.h.* and *C. ad.* antibody, whereas ACP demonstrated no protection at a ratio of 1:2.4. These are compared to a control clot occurring at 34 sec. At a ratio of 1:4.8 venom, ACP provided only weak protection, with a clot occurring at 51 sec.

Cytotoxicity results revealed good protection against cellular destruction by purified SRA (Table 5). ACP also protected against cytotoxicity from *C. ad.* venom but required almost three times the SRA dose.

The ability of ACP and the SRA to mitigate the hemorrhagic effects of the individual venoms was evident in most every animal studied. The greatest protection was shown in the cases of *C. ad.* and *C. v.h.* with the species specific antivenins. Cross protection tests were slightly less protective in the cases of *C. at.* and *C. s.s.* In each test animal, the SRA was more effective than the ACP, when specific venom-antivenin combinations were studied, and in many cases species specific cross protection was evident. BSA afforded no protection. Fig. 3 is representative of the hemorrhagic results, showing the protective effects of SRA compared to ACP and BSA.

Preliminary guinea pig sensitization studies (Table 6) demonstrated that hypersensitivity reactions occurred in the guinea pigs challenged with ACP and BSA but no reactions occurred in the animal challenged with SRA. These tests are being repeated in larger animal groups to confirm this preliminary data. *In vitro* leukocyte histamine release to antigenic stimulus demonstrated the potential for acute hypersensitivity reaction. Table 7 summarizes *in vitro* data of a patient who

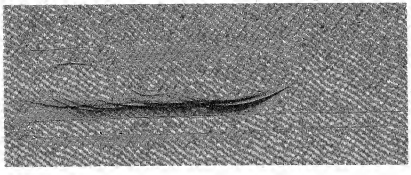

FIGURE 1: Immunoelectrophoresis of ACP (top light bands) and SRA, C. *adamanteus*, (bottom dark bands) developed with C. *adamanteus* venom.

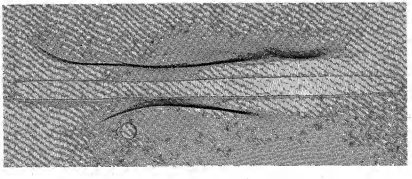

FIGURE 2: Immunoelectrophoresis of ACP (top dark bands) and SRA, C. *atrox*, (bottom single band) developed with rabbit anti-horse serum.

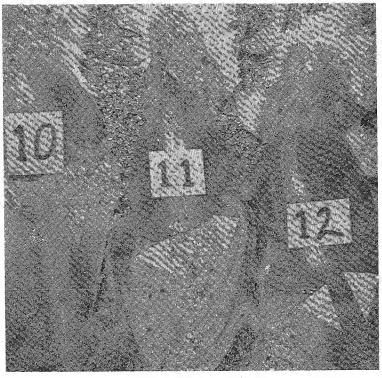

FIGURE 3: Results of hemorrhagic study: Number 10 shows ACP on left with BSA on right (arrow points to large hemorrhagic areas with both); Number 11 shows BSA on left with SRA on right; note lack of hemorrhage with SRA; Number 12 shows both ACP and BSA showing large hemorrhages.

suffered anaphylaxis to a horse serum skin test after being envenomated by a rattlesnake. These data indicate that this patient is very sensitive to foreign antigens with a spontaneous histamine release of 55% in standard human plasma. Base line histamine release in this patient was 8%, when his leukocytes were incubated in his own plasma. SRA antigenic stimulation caused a 12% histamine release from leukocytes, whereas ACP caused a 50% release and horse serum caused a 55% release.

TABLE 1: Protein assay

Antivenin 200 µg/ml	% Purity
C. atrox	77
C. adamanteus	74.4
C. viridis helleri	89.5
C. scutulatus scutulatus	77.8
ACP	61.8

TABLE 2: Lethality determinations of *Crotalus adamanteus* venom and antisera

Product	No. mice	No. dead	No. alive
ACP	10	8	2
SRA, *C. ad.*	10	0	10
SRA, *C. at.*	10	8	2
SRA, *C. v.h.*	10	7	3
SRA, *C. s.s.*	10	6	4
BSA	10	10	0

TABLE 3: Lethality determinations of *Crotalus viridis helleri* venom and antisera

Product	No. mice	No. dead	No. alive
ACP	10	9	1
SRA, *C. ad.*	10	4	6
SRA, *C. at.*	10	8	2
SRA, *C. v.h.*	10	1	9
SRA, *C. s.s.*	10	8	2
BSA	10	10	0

DISCUSSION: Preliminary investigation of the neutralization capacities of SRA and ACP involved lethality protection, fibrinogen clot prevention, prevention of cytotoxicity, protection against hemorrhage and sensitivity studies. These investigations, although not complete, indicate that the purified antivenin (SRA) isolated by affinity chromatography is more efficacious than the commercially available antivenin. The data on sensitivity reactions are not complete and detailed immunological studies are being conducted; however, our preliminary data indicated that the purified SRA would be less likely to produce acute anaphylaxis in individuals sensitive to horse serum than would ACP. Also, due to the removal of

TABLE 4: Fibrinogen clotting results

C. ad. venom (5 mg/ml)	Antivenin	Ratio	Clotting time
1) 0.1 ml	Control buffer	--	34 sec
2) 0.1 ml	0.2 ml *C. ad.* AB (6 mg/ml)	1:2.4	No clot
3) 0.1 ml	0.2 ml *C. v.h.* AB (5 mg/ml)	1:3	No clot
4) 0.1 ml	0.2 ml ACP (6 mg/ml)	1:2.4	33 sec
5) 0.1 ml	0.4 ml ACP (6 mg/ml)	1:4.8	51 sec

TABLE 5: Cytotoxicity studies

Tissue culture: Embryonic lung		
Venom	Antivenin	30 min post-incubation results
C. v.h. 20 µg	--	4+ Cytotoxicity
C. v.h. 20 µg	*C. v.h.* 89.5 µg	No Cytotoxicity
C. v.h. 20 µg	ACP 247.2 µg	No Cytotoxicity
C. ad. 50 µg	--	4+ Cytotoxicity
C. ad. 50 µg	*C. ad.* 74.4 µg	2+ Cytotoxicity
C. ad. 50 µg	*C. ad.* 93.0 µg	No Cytotoxicity
C. s.s. 60 µg	--	4+ Cytotoxicity
C. s.s. 60 µg	*C. s.s.* 140.0 µg	No Cytotoxicity

extraneous foreign protein, the incidence of serum sickness reactions should be significantly reduced.

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TABLE 6: Guinea pig sensitization

Guinea pig	Challenging agent/dose	Result
#1	ACP 1 mg	Sneezing, scratching, difficulty breathing; survived
#2	ACP 1 mg	Sneezing, scratching; survived
#3	SRA (*C. v.h.*) 1 mg	No reaction
#4	BSA 1 mg	Death in 5 min

TABLE 7: Isotopic histamine release assay from basophils

Patient's cells	% Release
PT cells - *St'd serum* + 25 µl buffer*	55
PT cells - *PT serum* + 25 µl buffer	8
PT cells - *PT serum* + 25 µl 1:10 horse serum	55
PT cells - *PT serum* + 25 µl ACP (1 mg/ml)	50
PT cells - *PT serum* + 25 µl purified *C. ad.* AB (1 mg/ml)	12

*TRIS buffer + Ca^{++} + Mg^{++}

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